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Characterization of Antimicrobial, Antioxidant, Cytotoxic Activity and Chemical Composition of *Punica granatum* Rind Extract

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Abstract

This study was carried out to characterize antimicrobial, antioxidant and cytotoxic activities of *Punica granatum* rind extract as well as its chemical composition. The main objective of the present study is to reveal the medicinal values of *P. granatum* rind. Antimicrobial property of *P. granatum* rind extract was revealed by using two fold broth micro dilution method against *Aeromonas hydrophila*, *Escherichia coli*, *Edwardsiella tarda*, *Flavobacterium* spp., *Klebsiella pneumonia*, *Salmonella typhi*, *Vibrio alginolyticus*, *V. parahaemolyticus*, *V. cholerae* and *Pseudomonas aeruginosa* whereas antioxidant activity of the plant extract was determined with α, α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging method. Cytotoxic activity of the plant extract was evaluated with Colorimetric MTT (tetrazolium) assay against human breast cell line (MCF-7). Chemical compounds of the plant extract were screened and identified by using gas chromatography-mass spectrometry (GC-MS). The results of the present study showed that the MIC values of *P. granatum* rind extract ranged from 3.91 to 15.63 mg/l against the tested bacterial isolates. The value of IC_{50} of the plant extract against DPPH and MCF-7 cell line was 0.144 ± 0.087 ppt and 0.54 ± 0.03 µg/ml, respectively. A total of 14 chemical compounds were successfully identified from P. granatum rind extract and the major compound was Hexanamide, N-phenyl (68.52%.). The findings of the present study indicate promising medicinal values of the plant extract.

1. Introduction

Punica granatum is a member of family Lythraceae and can be found widely in the tropical and subtropical areas such as Turkey, Iran, United States and Asian countries including Malaysia.. However, *P. granatum* is not a native plant in Malaysia but can grow well and became popular among farmer due to high demand of its fruit from market. It is well known and widely used as traditional medicine and its fruit was used for food for thousand years ago (Saleheddin & Kader, 1984). It became an important commodity in most of the countries such as Iran with the annual production of this fruit is around 700,000 tons. The plant was also used in the preparation of tinctures and cosmetic (Finkel & Holbrook, 2000). *P. granatum* possesses anti-inflammatory activity and popular in the western world. In addition, *P. granatum* fruit and flower were used for the treatment of inflammatory diseases in Asia and Europe (Shukla at al., 2008). Constituents of *P. granatum* were also well documented. For instance, pomegranate juice possesses anthocyanins, ascorbic acid, quercetin and rutin (Jurenka, 2008). On the other hand, pomegranate seed oil, pericarp, leaves, flower, root, and bark contain compounds such as sterols, tannins, gallic acid and ellagitannins (Jurenka, 2008). Although the studies of medicinal values of *P. granatum* were well established, findings from the present study may add values to the literature by investigating the antimicrobial, antioxidant and cytotoxic activities of *P. Granatum* rind extract against bacterial isolates from aquatic animals.

2. Materials and Methods

2.1. Plant Material

Pomegranate (*P. granatum*) fruits was purchased from an orchard located at Pasir Puteh, Kelantan, Malaysia. Samples of fresh pomegranate rind were oven-dried at 37°C for 4 days and milled into fine powder. Next, the plant sample was freeze dried prior to extraction using 70% methanol and concentrated at 1 g/ml. Finally, the plant extract was kept in -20°C until further use (Lee & Wendy, 2011).

2.2. Bacteria Isolates

Bacterial isolates in present study included *A. hydrophila*, *E. tarda*, *E. coli*, *Flavobacterium sp.*, *Klebsiella sp.*, *P. aeruginosa*, *Salmonella sp.*, *V. alginolyticus*, *V. cholerae* and *V. parahaemolyticus* were isolated from various aquatic animals, identified and kept in tryptic soy agar (TSA) for further uses.

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2.3. Minimum Inhibitory Concentration (MIC) Determination

The values of minimum inhibitory concentration (MIC) of *P. granatum* rind extract against bacterial isolates were determined through a two-fold broth micro dilution method (Lee & Wendy, 2011). The bacterial isolates were cultured in tryptic soy broth for 24 h at room temperature and the concentration of these cultures were adjusted to 10^9 CFU ml by using physiological saline. The concentration was cross check with a Biophotometer (Eppendorf, Germany). The bacterial suspensions were then inoculated into a microtiter plate that contained a serial dilution of *P. granatum* rind extract and positive control. The microplate was then incubated at room temperature for 24 h. The MIC values were defined as the lowest concentration of the *P. granatum* rind extract and positive control in the wells of the microtiter plate that showed no visible turbidity after 24 h incubation (Lee et al., 2011).

2.4. Antioxidant Assay

DPPH radical scavenging method was conducted as described by Gadow et al., 1997 with some modifications. The assay was carried out in a 96 wells ELISA plate with three replicates. Twenty microliter of the sample solution at various concentration by 2-fold dilutions from 10 mg/ml to 0.156 mg/ml was added into the wells followed by the addition of 200 µl methanolic DPPH (Sigma, Germany) solution (6 x 10-5 M) into all wells. Quercetin (Sigma, Germany) was used as positive control at 1 mg/ ml. Next, the sample were left in dark for 30 minutes incubation period. The absorbance value of the sample and the blank (well containing 20 mL DMSO) was recorded by using ELISA reader at 515 nm. The inhibition percentage of the DPPH radical scavenging activity was calculated based on the following formula:

$$\% \text{ Inhibition} = [(\text{Abs blank} - \text{Abs sample}) / \text{Abs blank}] \times 100$$

where, Abs blank is the absorbance of DPPH of the well containing DMSO and Abs sample is the absorbance of the sample after 30 minutes.

2.5. Cytotoxic Assay

The human breast (MCF-7) cell line was derived from ATCC (USA). All the cells were grown in standard cell medium (RPMI 1640) supplemented with 5% fetal bovine serum in a 5% CO_2 atmosphere at 37°C. The cells were then transferred into microplate at the concentration of 1 X 10^6 cells per well for cytotoxicity test of the plant extract. After 48 h, cell proliferation was measured by MTT assay. MTT (3-(4, 5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma, USA) assay was carried out as described by Mosmann (1983). 10 µl of MTT solution (5 mg/ml in PBS) was added to each well of 96-well micro plate followed by 4 h incubation at 37 °C. Next, MTT solution was removed and 150 µl acid isopropanol was added to each well to dissolve the dark blue crystals. Reading of the absorbance which correlated with the percentage of cell viability in the micro plate was performed using an ELISA reader at wavelength 570 nm within 1 h after the addition of acid isopropanol and agitation with an orbital shaker (Lee et al., 2012).

The IC_{50} value was calculated from the following formula as described in the previous study (Adebayo et al., 2010):

$$\log_{10}(IC_{50}) = \frac{\log_{10} C_L (I_H - 50) + \log_{10} C_H (50 - I_L)}{I_H - I_L}$$

$IC_{50} = 10\log_{10}(IC_{50})$

Where:

I_H : I% above 50%

I_L : I% below 50%

C_H : High drug concentration

C_L : Low drug concentration

2.6. Phytochemical Analysis

The chromatographic procedure was carried out using a Shimadzu QP2010-GC-MS with autosampler. The sample was diluted 25 times with acetone and 1 µl of sample was injected into a column. A fused silica capillary column HP5-MS (30 mm x 0.32 mm, film thickness 0.25 µm) was used. Helium was the carrier gas, and a split ratio of 1/100 was used. The oven temperature used was maintained at 60°C for 8 min. The temperature was then gradually raised at a rate of 3°C per min to 180°C and maintained at 180°C for 5 min. The temperature at the injection port was 250°C. The components of the test solution were identified by comparing the spectra with those of known compounds stored in internal library (Lee et al., 2016).

3. Results and Discussion

The MIC values of *Punica granatum* rind extract ranged from 3.91 to 15.63 mg/l in which the plant extract at the concentration of 3.91 mg/l can inhibit the growth of *Edwardsiella tarda*, *Escherichia coli*, *Flavobacterium* sp., *Pseudomonas aeruginosa* and *Vibrio cholera*e whereas at the concentration of 7.81 mg/l of the plant extract, *Aeromonas hydrophila*, *Klebsiella* sp., *Salmonella* sp. and *Vibrio alginolyticus* were failed to grow. The plant extract was able to control the growth of *V. Parahaemolyticus* at the concentration of 15.63 mg/l (Table 1). The value of IC_{50} of the plant extract against DPPH and MCF-7 cell line was 0.144 ± 0.087 ppt and 0.54 ± 0.03 µg/ml, respectively. A total of 14 chemical compounds were successfully identified with the major compound was Hexanamide, N-phenyl 68.52%. This was followed by Octaethylene glycol 7.11%, Butenoic acid 4.73%, 2, 5-dioxoimidazolidine 3.30%, Hexagol 2.52%, 15-Crown-5 2.09%, 1, 4, 7, 10, 13, 16-Hexaoxacyclooctadecane 2.00%, 4-Mercaptophenol 1.52%, Vitamin E 0.42%, Acetic acid 0.36%, Propanoic acid 0.33%, 2-Furanmethanol 0.11%, Oxalic acid 0.04%, Aniline 0.04% and another 10 unidentified compounds 6.91% (Table 2)..

The studies of antimicrobial property of *P. granatum* were well documented in the literature. For instance, Salgado et al. (2009) claimed that the fruit of *P. granatum* possesses inhibitory activity against *Pseudomonas aeruginosa, Escherichia coli, Enteroccocus faecalis, Enterobacter aerogenes, Staphyloccocus aureus, Micrococcus luteus* and *Bacillus* sp. Another study that claimed the antimicrobial property of *P. granatum* was Duman et al. (2009) in which they revealed that *P.*

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granatum was able to inhibit the growth of seven species of bacteria (*Bacillus megaterium* DSM 32, *Pseudomonas aeruginosa* DSM 9027, *Staphylococcus aureus* Cowan 1, *Corynebacterium xerosis* UC 9165, *Escherichia coli* DM, *Enterococcus faecalis* A10, *Micrococcus luteus* LA 2971) and three fungi (*Kluvyeromyces marxianus* A230, *Rhodotorula rubra* MC12, *Candida albicans* ATCC 1023). Similar finding was observed in the present study in which all the bacterial isolates were sensitive to the plant extract. In so far, the antimicrobial works of *P. granatum* were concerned on the human pathogen. Therefore, this is the first report on the antimicrobial activity of *P. granatum* rind extract against pathogens isolated from aquatic animals. Hence, we may conclude that *P. granatum* possesses antimicrobial property against pathogen from various hosts. Furthermore, chemical compounds such as Hexanamide, N-phenyl, Butenoic acid, Acetic acid, Propanoic acid and Oxalic acid that found in the present study may play role to the antimicrobial activity of the plant extract.

Yasoubi et al. (2007) reported that *P. granatum* possesses antioxidant activity against butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). Other studies by Singh et al. (2002) as well as Ricci et al. (2006) claimed that this plant showed the inhibitory activity against DPPH. Similar finding was observed in the present study. This was supported by the finding of Kishore et al. (2009) that claimed this plant extract can be use as embryo protector.

The potential of *P. granatum* to be as cytotoxic agent is promising. Several studies have revealed the cytotoxic property of *P. granatum* such as Lansky et al. (2006). It was claimed that *P. granatum* showed inhibitory to PC-3 prostate cancer cell line. Another study by Kawaii & Lansky (2004) revealed that *P. granatum* possesses cytotoxic activity against HL-60, human promyelocytic leukemia cells. In the present study, compounds such as Hexanamide, N-phenyl, Octaethylene glycol, Butenoic acid, Hexagol, 4-Mercaptophenol, Acetic acid, Propanoic acid and Oxalic acid found in P. granatum rind extract could have contribute to the cytotoxic activity.

Another important finding of the present study was that *P. granatum* has great potential to be used as drug to combat against mosquito. Pridgeon et al. (2010) claimed that hexanamide was toxic to *Aedes aegypti* L. in which hexanamide was the major compound in the plant extract. Therefore, the plant extract can be further studied for the use as mosquito repellent.

4. Conclusion

In conclusion, antimicrobial, antioxidant and cytotoxic properties of *P. granatum* rind extract was found to be effective in controlling bacterial isolates from aquatic organisms.

Table 1: Minimum inhibition concentration (MIC) of *Punica granatum* rind extract against bacterial isolates

Bacterial isolates	MIC (mg/l)
Aeromonas hydrophila	7.81±0.00
Edwardsiella tarda	3.91±0.00
Escherichia coli	3.91±0.00
Flavobacterium sp.	3.91±0.00
Klebsiella sp.	7.81±0.00
Pseudomonas aeruginosa	3.91±0.00
Salmonella sp.	7.81±0.00
Vibrio alginolyticus	7.81±0.00
Vibrio cholera	3.91±0.00
Vibrio parahaemolyticus	15.63±0.00

Table 2: Compound composition of *Punica granatum* rind extract

Compound	Compound Composition (%)
Hexanamide, N-phenyl	68.52
Octaethylene glycol	7.11
Butenoic acid	4.73
2, 5-dioxoimidazolidine	3.30
Hexagol	2.52
15-Crown-5	2.09
1, 4, 7, 10, 13, 16-Hexaoxacyclooctadecane	2.00
4-Mercaptophenol	1.52
Vitamin E	0.42
Acetic acid	0.36
Propanoic acid	0.33
2-Furanmethanol	0.11
Oxalic acid	0.04
Aniline	0.04
10 unidentified compounds	6.91
Total	100.00

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